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ProteoStat® Aggresome Detection Kit

for flow cytometry and fluorescence microscopy

Instruction Manual

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For research use only.

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I. Introduction

In mammalian cells, aggregated proteins may be concentrated by microtubule-dependent retrograde transport to perinuclear sites of aggregate deposition, referred to as aggresomes. Aggresomes are inclusion bodies that form when the ubiquitin–proteasome machinery is overwhelmed with aggregation-prone proteins.⁽¹⁾ Typically, an aggresome forms in response to some cellular stress, such as hyperthermia, viral infection or exposure to reactive oxygen species. Aggresomes appear to provide a cytoprotective function by sequestering the toxic, aggregated proteins and may also facilitate their ultimate elimination from cells by autophagy. Certain cellular inclusion bodies associated with human disease are thought to arise from an aggresomal response, including Lewy bodies associated with neurons in Parkinson's disease, Mallory bodies associated with liver cells in alcoholic liver disease and hyaline inclusion bodies associated with astrocytes in amyotrophic lateral sclerosis.⁽²⁾

Non-physiological protein mutations or genetically engineered cell lines have been developed for assessment of the effects of protein aggregation within cells.⁽³⁾ Enzo Life Sciences' ProteoStat® Aggresome Detection Kit contains a novel 488 nm excitable red fluorescent molecular rotor dye to specifically detect denatured protein cargo within aggresomes and aggresome-like inclusion bodies in fixed and permeabilized cells. The detection reagent supplied in the ProteoStat® Aggresome Detection Kit becomes brightly fluorescent upon binding to aggregated proteins within vesicles produced during aggresome formation and has been validated under a wide range of conditions in which the autophagy and proteasome pathways are known to be modulated. MG-132, a proteasome inhibitor, is included as a positive control in the kit. A nuclear counterstain is provided in the kit as well to highlight this organelle. This kit can be used to facilitate understanding of the basic molecular processes involved in the four key steps of autophagosome-dependent degradation, namely induction or cargo packaging, vesicle formation and completion, docking and fusion, and vesicle breakdown. The assay is potentially applicable to the identification of small molecules that inhibit aggresome formation as well as immuno-localization studies between aggregated protein cargo and the various proteins implicated in aggresome formation, such as histone deacetylase 6, parkin, ataxin-3, dynein motor complex and ubiquilin-1.

II. Reagents Provided and Storage

All reagents are shipped on dry ice. Upon receipt, the kit should be stored at $\leq 20^{\circ}\text{C}$, protected from light. When stored properly, these reagents are stable for one year from date of receipt. **Avoid repeated freezing and thawing.**

Reagents provided in the kit are sufficient for approximately 100 assays for flow cytometry application or 200 assays for fluorescence microscopy.

Reagent	Quantity
ProteoStat [®] Aggresome Detection Reagent	10 μL
Hoechst 33342 Nuclear Stain	50 μL
Proteasome Inhibitor (MG-132)	120 nmol
10X Assay Buffer	25 mL

III. Additional Materials Required

- Flow cytometer equipped with 488 nm laser source
- Standard fluorescence microscope
- Tubes appropriate for holding cells for the flow cytometer
- Calibrated, adjustable precision pipettors, preferably with disposable plastic tips
- Adjustable speed centrifuge with swinging buckets (for suspension cultures)
- Deionized water
- Anhydrous DMSO
- Total growth medium suitable for cell type
- Glass microscope slides
- Glass cover slips (18 x 18 mm)
- Paraformaldehyde
- EDTA, pH 8
- Triton X-100
- 1X Phosphate buffered saline (1X PBS)

IV. Safety Warnings and Precautions

- This product is for research use only and is not intended for diagnostic purposes.
- The ProteoStat[®] Aggresome Detection Reagent contains DMSO which is readily absorbed through the skin. DMSO is harmful if ingested or absorbed through the skin and may cause irritation to the eyes. Observe appropriate precautions when handling these reagents.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
- To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.

V. Methods and Procedures

The procedures described in this manual assume that the user is familiar with the basic principles and practices of flow cytometry and is able to run samples according to the operator's manual pertaining to the instrument being used.

NOTE: *Allow all reagents to thaw at room temperature before starting with the procedures. Upon thawing, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution. Briefly centrifuge the vials at the time of first use, as well as for all subsequent uses, to gather the contents at the bottom of the tube.*

A. REAGENT PREPARATION

1. Positive Control

MG-132 has been shown to be a regulator of protein homeostasis inducing both the unfolded protein response and the heat shock response.^(4, 5) Proteasome inhibition induces accumulation of heat shock protein mRNA, activation of heat-shock proteins, and enhanced thermotolerance in various cell types. Inhibiting proteasomes accelerates the formation of perinuclear aggresomes within cells.

Reconstitute the lyophilized MG-132 (120 nmol) in 12 μ L DMSO for a 10 mM stock solution. To use it as a positive control, dilute the MG-132 to 5-10 μ M into your culture medium and grow cells for 6 to 18 hours. The agent has been validated with human cervical carcinoma cell line, HeLa, human T-lymphocyte cell line, Jurkat, and human bone osteosarcoma epithelial cell line, U2OS. Unused stock solution of MG-132 may be stored at -20°C for several weeks.

2. 1X Assay Buffer

Allow the 10X Assay Buffer to warm to room temperature. Make sure that the reagent is free of any crystallization before dilution. Prepare enough 1X Assay Buffer for the number of samples to be assayed by diluting each milliliter (mL) of the 10X Assay Buffer with 9 mL of deionized water.

3. 4% Formaldehyde Solution

The following procedure is for preparation of 10 mL of 4% formaldehyde solution: Dilute 0.4 gram paraformaldehyde to a final volume of 10 mL with 1X Assay Buffer. Mix well.

4. Permeabilizing Solution

The following procedure is for preparation of 10 mL of permeabilizing solution (0.5% Triton X-100, 3 mM EDTA, pH 8): Add 50 μ L Triton X-100 and 60 μ L of 0.5M EDTA, pH 8, to 9.89 mL of 1X Assay Buffer. Mix well.

5. ProteoStat® Aggresome Detection Reagent

For optimal staining, the concentration of the ProteoStat® Aggresome dye will vary depending upon the application. Suggestions are

provided to use as guidelines, though some modifications may be required depending upon the particular cell type employed in the application.

- a. **For fluorescence microscopy**, prepare a sufficient amount of Dual Detection Reagent for the number of samples to be assayed as follows: For every 2 mL of 1X Assay Buffer (see section V-A2, page 3) or cell culture medium, add 1 μ L of ProteoStat® Aggresome Detection Reagent and 2 μ L of Hoechst 33342 Nuclear Stain.

NOTE: (a) *The dyes may be combined into one staining solution or each may be used separately, if desired.*

(b) *The Hoechst 33342 Nuclear Stain can be diluted further if its staining intensity is much stronger than that of the ProteoStat® aggresome red dye.*

(c) *When staining BFP- or CFP-expressing cells, the Hoechst 33342 Nuclear Stain should be omitted due to its spectral overlap with these fluorescent proteins.*

- b. **For flow cytometry**, dilute the ProteoStat® Aggresome Detection Reagent 5,000-10,000 fold with 1X Assay Buffer or buffer of choice.

B. CELL PREPARATIONS

Cells should be maintained via standard tissue culture practices. Always make sure that cells are healthy and in the log phase of growth before using them for the experiment.

Positive control cells should be pretreated with the MG-132, a cell-permeable proteasome inhibitor, for 6~18 hours. Response to MG-132 is time and concentration dependent and may also vary significantly depending upon cell type and cell line. Negative control cells should be treated with a vehicle (DMSO, media or other solvent used to reconstitute or dilute an inducer or inhibitor) for an equal length of time under similar conditions.

C. CELL ANALYSIS BY FLUORESCENCE/CONFOCAL MICROCOPY (ADHERENT CELLS)

1. Grow the cells directly on the glass slides, to a ~80% confluency.
2. Treat the cells with the compound(s) of interest, and negative control cells with vehicle only.
3. Prepare positive control cells by incubating with the diluted Proteasome Inhibitor (5-10 μ M MG-132, see section V-A1, page 3) for 18 hours under normal tissue culture conditions.
4. Carefully wash the cells twice with 200 μ L of 1X PBS per 1 cm^2 surface area for each wash. a typical 2-to-4-well slide would require ~ 100 μ L).
5. Carefully remove excess 1X PBS and dispense 200 μ L of 4% formaldehyde per 1 cm^2 surface area. Incubate for 30 minutes at room temperature.

6. Carefully wash the cells twice with 200 μ L of 1X PBS per 1 cm^2 surface area for each wash.
7. Carefully remove excess 1X PBS and dispense 120 μ L of Permeabilizing Solution (see section V-A4, page 3) per 1 cm^2 surface area. Place slides on ice, and gently shake them for 30 minutes.
8. Carefully wash the cells twice with 200 μ L of 1X PBS per 1 cm^2 surface area for each wash.
9. Carefully remove excess buffer and dispense 200 μ L of Dual Detection Reagent (see section V-A5a, page 4) per 1 cm^2 surface area to cover the monolayer cells.
10. Protect samples from light and incubate for 30 minutes at room temperature.
11. Carefully wash the cells with 200 μ L of 1X PBS per 1 cm^2 surface area. Remove excess buffer and place coverslip on microscope slide.
12. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification recommended). Use a standard Texas Red filter set for imaging the cell aggresome signal and a DAPI filter set (optional) for imaging the nuclear signal.

D. CELL ANALYSIS BY FLUORESCENCE/CONFOCAL MICROSCOPY (SUSPENSION CELLS)

1. Cells should be cultured to a density not to exceed 1×10^6 cells/mL. Make sure that cells are healthy and in the log phase of growth before starting an experiment.
2. Treat the cells with the compound of interest and the negative control cells with vehicle.
3. Prepare positive control cells by incubating with the diluted Proteasome Inhibitor (5-10 μ M MG-132, see section V-A1, page 3) for 18 hours under normal tissue culture conditions.
4. At the end of the treatment, collect cells to ensure cell count of 2×10^5 to 4×10^5 cells/sample. Centrifuge suspension for 5 minutes at room temperature at 400 x g.
5. Remove the supernatant and carefully re-suspend the cells in 2 mL of 1X PBS.
6. Centrifuge for 5 minutes at room temperature at 400 x g. Carefully remove most of the supernatant by aspiration. Using a micropipet, gently re-suspend the cell pellet in the remaining 1X PBS (~ 200 μ L).
7. Using a micropipet, add drop wise the cell suspension into 2mL of 4% formaldehyde solution (see section V-A3, page 3) contained in a 15 mL conical tube. **Slowly** vortex the fixative during addition of the cell suspension. Allow to stand for 30 minutes at room temperature.
8. Collect the fixed cells by centrifugation at 800 x g for 10-15 minutes.
9. Pour out the supernatant. **Do not** aspirate.

CAUTION: Fixed cells do not stick to the walls of the tube as tightly as live cells. Be careful not to lose the cell pellet.

10. Re-suspend the cell pellet in the remaining small volume of supernatant using a micropipet. Add 2 mL of 1X PBS to the cells, mix well and centrifuge at 800 x g for 10-15 minutes. Pour out the super-

natant, being careful not to lose the cell pellet.

11. Re-suspend the cell pellet in the remaining small volume of supernatant using a micropipet. Using a micropipet, add drop wise the cell suspension into 2 mL of permeabilizing solution (see section V-A4, page 4) contained in a 15 mL conical tube. **Slowly** vortex the permeabilizing solution during addition of the cell suspension. Allow to incubate for 30 minutes on ice.
12. Collect the fixed cells by centrifugation at 800 x *g* for 10-15 minutes. Pour out the supernatant, being careful not to lose the cell pellet.
13. Re-suspend the cell pellet in the remaining small volume of supernatant using a micropipette and wash again using 2 ml 1X PBS. Then, centrifuge at 800 x *g* for 10-15 minutes. Pour out the supernatant, being careful not to lose the cell pellet.
14. Re-suspend the cell pellet in 100 μ L of Dual Detection Reagent (from step V-A5a, page 4). It is important to achieve a monodisperse cell suspension at this step by gently pipetting up and down repeatedly.
15. Protect samples from light and incubate for 30 minutes at room temperature.
16. Resuspend the cells in 2 mL of 1X PBS, centrifuge them at 800 x *g* for 10 - 15 minutes and remove the supernatant.
17. Resuspend the cells in 100 μ L of 1X PBS and apply a 20 μ L aliquot of the cell suspension, sufficient for 2×10^4 cells, onto a microscope slide. Immediately overlay the cells with a cover slip.
18. Analyze the stained cells by wide-field fluorescence or confocal microscopy (60X magnification recommended). Use a standard Texas Red filter set for imaging the cell aggresome signal and a DAPI filter set (optional) for imaging the nuclear signal.

E. CELL ANALYSIS BY FLOW CYTOMETRY (BOTH SUSPENSION AND ADHERENT CELL LINES)

1. Cells should be maintained via standard tissue culture practice in a humidified incubator at 37°C, 5% CO₂. Make sure that cells are healthy and in the log phase of growth before starting an experiment.
2. Treat cells with compound of interest and negative control cells with vehicle.
3. Prepare positive control cells by incubating with the diluted Proteasome Inhibitor (5-10 μ M MG-132, see section V-A1, page 3) for 18 hours under normal tissue culture conditions.
4. At the end of the treatment, trypsinize (adherent cells), or collect cells (suspension cells). Samples may contain 1×10^6 to 2×10^6 cells per mL.
5. Centrifuge at 400 x *g* for 5 minutes to pellet the cells. Carefully re-suspend and wash the cells with 2 mL of 1X PBS.
6. Remove the supernatant and carefully re-suspend the cells in 2 mL 1X PBS.
7. Centrifuge for 5 minutes at room temperature at 400 x *g*. Carefully remove most of the supernatant by aspiration. Using a micropipet,

gently re-suspend the cell pellet in the remaining 1X PBS (~ 200 µL).

8. Using a micropipet, add drop wise the cell suspension into 2mL of 4% formaldehyde solution (see section V-A3, page 3) contained in a 15 mL conical tube. **Slowly** vortex the fixative during addition of the cell suspension. Allow to stand for 30 minutes at room temperature.
9. Collect the fixed cells by centrifugation at 800 x g for 10-15 minutes.
10. Pour out the supernatant. **Do not** aspirate.

CAUTION: *Fixed cells do not stick to the walls of the tube as tightly as live cells. Be careful not to lose the cell pellet.*

11. Re-suspend the cell pellet in the remaining small volume of supernatant using a micropipet. Add 2 mL of 1X PBS to the cells, and centrifuge at 800 x g for 10-15 minutes. Pour out the supernatant, being careful not to lose the cell pellet.
12. Re-suspend the cell pellet in the remaining small volume of supernatant using a micropipet. Using a micropipet, add drop wise the cell suspension into 2 mL of permeabilizing solution (see section V-A4, page 3) contained in a 15 mL conical tube. **Slowly** vortex the permeabilizing solution during addition of the cell suspension. Allow to incubate for 30 minutes on ice.
13. Collect the fixed cells by centrifugation at 800 x g for 10-15 minutes. Pour out the supernatant, being careful not to lose the cell pellet.
14. Wash the cells by re-suspending the cell pellet with 2mL 1X PBS using a micropipet. Then, transfer the cell suspension through the cap of a BD tube (with cell strainer cap, BD # 352235) to remove cell debris. Then, centrifuge the tube at 800 x g for 10-15 minutes. Remove the strainer cap and carefully pour out the supernatant, being careful not to lose the cell pellet.
15. Re-suspend the cell pellet in 500 µL of freshly diluted (5,000 - 10,000 -fold) ProteoStat® Aggresome Red Detection Reagent (see step V-A5b, page 4). It is important to achieve a monodisperse cell suspension at this step by gently pipetting up and down repeatedly.
16. Protect samples from light and incubate for 30 minutes at room temperature.
17. Analyze the samples in the FL3 channel of a flow cytometer. No washing is required prior to the flow cytometry analysis.
18. CALCULATION OF RESULTS:
 - a. Obtain the mean fluorescence intensity (MFI) values for treated and untreated sample.
 - b. Calculate the aggresome propensity factor (APF) value using the following formula:

$$APF = 100 \times (MFI_{\text{treated}} - MFI_{\text{control}}) / MFI_{\text{treated}}$$

The expected APF value using the control MG-132 is greater than 25 .

VI. APPENDICES

A. FLUORESCENCE CHANNEL SELECTION FOR DATA COLLECTION

The selection of optimal filter sets for a fluorescence microscopy application requires matching the optical filter specifications to the spectral characteristics of the dyes employed in the analysis (see Figure 1). Consult the microscope or filter set manufacturer for assistance in selecting optimal filter sets for your microscope. Predesigned filter sets for Texas Red should work well for this application.

For flow cytometry, fluorescence channel FL-3 is recommended for analysis of the ProteoStat[®] aggresome red dye staining using a 488 nm laser source.

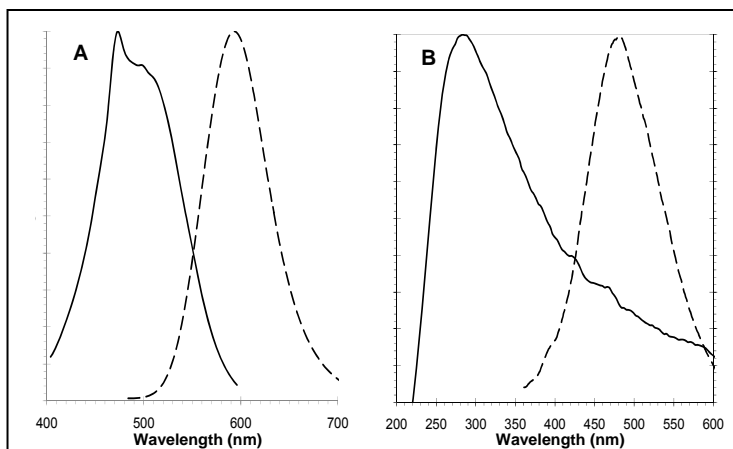


Figure 1. Excitation and fluorescence emission spectra for ProteoStat[®] Aggresome dye, ex/em 500/600 nm (panel A) and Hoechst 33342, ex/em 350/461 nm (panel B). All spectra were determined in 1X Assay Buffer.

B. EXPECTED RESULTS

1. Microscopy

Selective degradation of intracellular targets, such as misfolded proteins and damaged organelles, is an important homeostatic function that autophagy has acquired in addition to its more general role in restoring the nutrient balance during stress and starvation. Although the exact mechanism underlying selection of autophagic substrates remains the subject of much study, ubiquitinylation is now appreciated to be a signal for autophagic degradation of aggregated proteins.

In order to better understand the nature and contribution of the pathway modulators to the specific targeting of protein cargo for degradation, the ProteoStat[®] aggresome red fluorescent molecular rotor dye has been developed specifically devised to detect aggregated proteins within the aggresome and aggresome-like structures. This detection reagent detects protein cargo accumulation within these structures, as observed by fluorescence microscopy (see Figure 2).

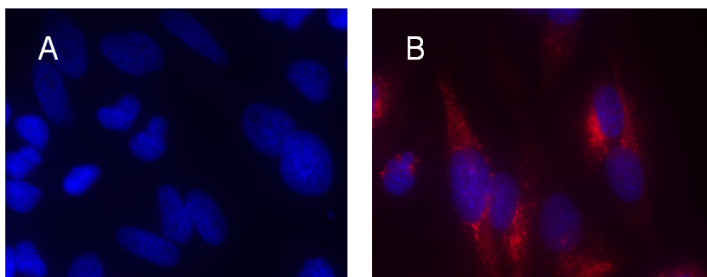


Figure 2. ProteoStat[®] aggresome dye detects protein accumulation within aggresomes, as observed by fluorescence microscopy. HeLa cells were mock-induced with 0.2% DMSO (panel A) or induced with 5 μ M MG-132 (panel B) for 12 hours at 37°C. After treatment, cells were incubated with ProteoStat[®] aggresome dye for 30 minutes.

The ability to detect aggresomes was demonstrated using various potent, cell permeable and selective proteasome inhibitors: Lactacystin, Epoxomicin and Bortezomib (US FDA-approved drug Velcade[®]), as shown in Figure 3.

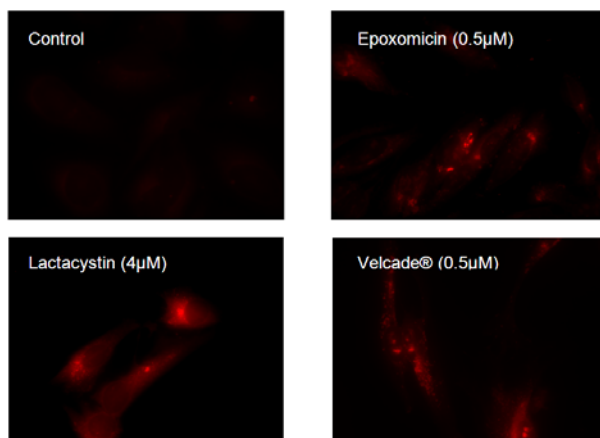


Figure 3. Aggresomes detected by ProteoStat[®] aggresome dye in HeLa cells after overnight incubation with various proteasome inhibitors, as observed by fluorescence microscopy.

The p62 protein (also called sequestosome 1 (SQSTM1)) is an ubiquitin-binding scaffold protein that co-localises with ubiquitinated protein aggregates in, for example, many neurodegenerative diseases and proteinopathies of the liver. The protein is able to polymerise *via* an N-terminal PB1 domain and can interact with ubiquitinated proteins *via* its C-terminal UBA domain.⁽⁶⁾ Also, p62 binds directly to LC3 and GABARAP family proteins *via* a specific sequence motif. The protein is itself degraded by autophagy and serves to link ubiquitinated proteins to the autophagic machinery in order to enable their degradation in the lysosome. Since p62 accumulates when autophagy is inhibited, and decreased levels can be observed when autophagy is induced, p62 may be used as a marker to study autophagic flux. Figure 4 shows the use of a fluorescein conjugated antibody directed to p62, in concert with the ProteoStat[®] aggresome dye, in co-localizing these elements of the autophagic process in intact cells. It is clear that p62 co-localizes with aggresomes.

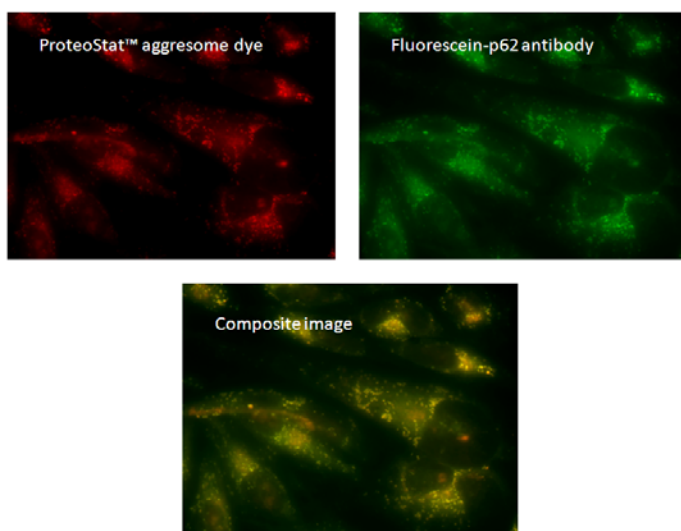
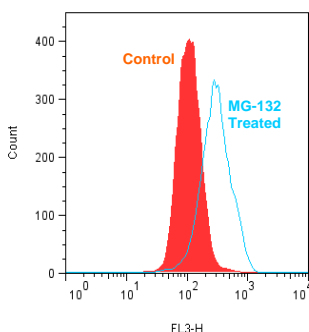


Figure 4. Aggresomes within HeLa cells, previously treated for 12 hours with 5 μ M MG-132, detected by ProteoStat[®] aggresome dye showing co-localization with fluorescein-p62 antibody, as observed by fluorescence microscopy.

2. Flow Cytometry

Figure 5 shows the typical results of flow cytometry-based analysis of cell populations using the ProteoStat® aggresome red detection kit. Uninduced control and 5 μ M MG-132-treated Jurkat cells (T-Cell leukemia) were used. After 18 hours treatment, cells were loaded with ProteoStat® aggresome red detection reagent, then analyzed without washing by flow cytometry. Results are presented by histogram overlays. Control cells were stained as well but display low fluorescence. In the samples treated with 5 μ M MG-132 for 18 hours. The ProteoStat® aggresome red dye signal increases over 2-fold, indicating that MG-132 induced aggresome formation in Jurkat cells. The APF value is about 66.



Treatment	Mean (FL3) Signal
Control	113
Treated (5 μ M MG-132)	335
Aggresome Propensity Factor (APF)	66

Figure 5. Flow cytometry-based cell aggresome analysis: Jurkat cells were mock-induced with 0.2% DMSO or induced with 5 μ M MG-132 for overnight hours at 37°C. After treatment, cells were fixed and incubated with ProteoStat® aggresome dye, then analyzed by flow cytometry without washing using a 488 nm laser with fluorescence detection in the FL3 channel. Results are presented as histogram overlays. In MG-132 treated cells, fluorescent green signal increases over 2-fold. The described assay allows assessment of the effects of protein aggregation.

VII. References:

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VIII. Troubleshooting Guide

Problem	Potential Cause	Suggestion
ProteoStat® aggresome red dye staining fails to stain live cells.	The dye is only suitable for fixed and/or permeabilized cells.	Use the dye only in fixed and/or permeabilized cells.
Low ProteoStat® aggresome red staining in all treatments, including positive control.	A low concentration of the ProteoStat® Aggresome Detection Reagent was used or the reagent was incubated with the cells for an insufficient length of time.	Either increase the reagent concentration or increase the incubation time.
	Cell viability is low.	Cells should be in log growth phase. Avoid shear stress, do not vortex, and avoid excessive formation of bubbles.
ProteoStat® aggresome red dye stained cells are too low to be readily quantified.	Cell density is either too low or cells were lost during processing.	Increase cell density. Fixed cells don't stick to walls of the tube as live cells. Follow the procedure described in the manual
Precipitate is observed in the 10X Assay Buffer	Precipitate forms at low temperatures.	Allow solution to warm to room temperature or 37° C, then vortex to dissolve all precipitate.
MG-132-treated cells appear dead or are no longer attached to the plate surface.	The EC ₅₀ of MG-132 may differ with different cell lines.	Try lowering the dose of MG-132.



www.enzolifesciences.com

Enabling Discovery in Life Science®

NORTH/SOUTH AMERICA

ENZO LIFE SCIENCES INTERNATIONAL, INC.

5120 Butler Pike

Plymouth Meeting, PA 19462-1202

USA

T 1-800-942-0430/(610) 941-0430

F (610) 941-9252

E info-usa@enzolifesciences.com

SWITZERLAND & REST OF EUROPE

ENZO LIFE SCIENCES AG

Industriestrasse 17, Postfach

CH-4415 Lausen

Switzerland

T +41/0 61 926 89 89

F +41/0 61 926 89 79

E info-ch@enzolifesciences.com

www.enzolifesciences.com

GERMANY

ENZO LIFE SCIENCES GMBH

Marie-Curie-Strasse 8

DE-79539 Lörrach

Germany

T +49/0 7621 5500 526

Toll Free 0800 664 9518

F +49/0 7621 5500 527

E info-de@enzolifesciences.com

www.enzolifesciences.com

BENELUX

ENZO LIFE SCIENCES BVBA

Melkerijweg 3

BE-2240 Zandhoven

Belgium

T +32/0 3 466 04 20

F +32/0 3 466 04 29

E info-be@enzolifesciences.com

www.enzolifesciences.com

UK & IRELAND

ENZO LIFE SCIENCES (UK) LTD.

Palatine House

Matford Court

Exeter EX2 8NL

UK

T 0845 601 1488 (UK customers)

T +44/0 1392 825900 (from overseas)

F +44/0 1392 825910

E info-uk@enzolifesciences.com

www.enzolifesciences.com

www.enzolifesciences.com

FRANCE

ENZO LIFE SCIENCES

c/o Covalab s.a.s.

13, Avenue Albert Einstein

FR -69100 Villeurbanne

France

T +33 472 440 655

F +33 437 484 239

E info-fr@enzolifesciences.com

www.enzolifesciences.com

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